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REMARKS

The Official Action mailed September 16, 2003, and the prior art relied upon therein have been carefully studied. The claims in the application are now claims 1-25, and these claims define novel and unobvious subject matter under §§ 102 and 103, and therefore should be allowed. Accordingly, the applicants hereby respectfully request favorable reconsideration allowance.

The Examiner notes that no reference to Goosen et al, listed as reference AD on the Form PTO/SB/08A filed as part of applicants' Information Disclosure Statement, has been received by the PTO. According to applicant's records, this document AD was indeed filed, but should have been referred to as "Sun et al" rather than "Goosen et al". The first named author is Anthony M. Sun, with Mattheus F. A. Goosen being the third named author. A duplicate copy of such document is attached herewith.

Applicants note that no rejections have been imposed under §112, and no criticisms have been made as to form. Applicants accordingly understand that the present application is deemed to be in good form and in full conformance with §112 by the PTO, and applicants are proceeding in reliance thereof.

Nevertheless, some amendments have been introduced in the claims to place the claims in better form for U.S. practice. For example, classical Markush language has been introduced into claims 6, 9, 10, 14 and 20. The word "step" has been either eliminated or replaced by the word "stage" for consistency. Capital (upper case) letters have been replaced by lower case letters at the beginning of each paragraph of claim 1, and additional minor amendments have been introduced in claim 1 to improve antecedent basis for later appearing terms. All such amendments are of a formal nature only, i.e. made to place the claims in better form consistent with U.S. practice. Such amendments are entirely cosmetic, i.e. are not narrowing amendments, because the scope of the claims has not been reduced. No limitations have been added and none are intended.

Claims 1-24 have been rejected under §103 as obvious from Lim et al USP 4,389,419 (Lim) in view of Francois et al USP 6,555,544 (Francois) and Kantor et al USP 4,895,725 (Kantor) and Patel et al USP 6,569,463 (Patel). This combination of four references to allegedly show obviousness is respectfully traversed.

Applicants wish to point out that the present invention results in a superior product as compared with the

prior art, in that a relatively large amount of lipophilic material can be incorporated into the microcapsule while retaining a high bioavailability (see specification page 3, lines 19-25; page 4, lines 9-12 and lines 23-28). The present invention involves a unique and non-obvious microencapsulation process, comprising the following steps:

1. Size reduction of the oily substance, in the presence of a surfactant (1st coating layer - by surfactant);
2. preparing a metal alginate solution and combining it with the solution of step 1;
3. dropwise addition of the solution of step 3 into a Ca^{+2} solution (2nd coating layer - by alginate);
4. rinsing the beadlets in an acid solution and drying; and
5. coating the beadlets with e.g. cellulose to obtain the microcapsule (3rd coating layer - e.g. by cellulose).

As pointed out in applicants' specification at page 4, lines 28 *et seq*, it would have been expected that a plurality of coating layers would adversely affect the bioavailability of the encapsulated compounds, but surprisingly this turns out to

be not so. This is a further unobvious aspect of the present invention.

The main reference relied upon, namely Lim, is not only acknowledged prior art, but is also incorporated by reference into applicants' specification, noting page 1, line 10. It forms the starting point of the present invention. Lim discloses what applicants' specification says it discloses. The PTO apparently agrees, as Lim has not been relied upon by itself, but only in combination with three other citations.

In particular, the rejection agrees that applicants' claim 1 differs from Lim in (1) "reducing the particle size of the lipophilic compound in the presence of a surfactant[,] and [(2)] in rinsing the beadlet in an acidic solution[,] and [(3)] in coating the beadlet." In other words, applicants' process as called for in claim 1 differs from Lim in stages (i), (v) and (vi), or in other words, providing the first and third coating layers.

Francois discloses a pharmaceutical composition suitable as a depot formulation for administration via intramuscular or subcutaneous injection comprising a solid ingredient suspended in water. As correctly stated in the rejection, Francois does teach the utilization of a surfactant to improve suspension of the particles of the solid active

ingredient in the water carrier, but the surfactants have nothing to do with reducing the size of the solid particles of active agent. It is also true as stated in the rejection that the particle size of the solid active agent is reduced by grinding as pointed out, for example, column 5, lines 29 and 30, and column 8, line 53. This of course has no relation to size reduction for a liquid.

Francois thus has nothing to do with the present invention, i.e. preparing a pharmaceutical composition suitable as a depot formulation for administration via intramuscular or subcutaneous injection, and comprising a finally ground active solid ingredient suspended in water, has nothing to do with the preparation of stable coated microcapsules. Perhaps even more importantly, Francois has nothing to do with Lim. The person of ordinary skill in the art of Lim, concerned with the production of microcapsules or vitamin encapsulation, as per Lim's art, would not even consider Francois (and if such skilled person were to consider Francois, that skilled person would learn nothing with respect to reducing the size of a vitamin oil). The proposed combination of Lim in view of Francois would not have been obvious to the person of ordinary skill in the art at the time the present invention was made.

Unlike Francois, Kantor at least relates to the same art as Lim and applicants. Kantor produces microcapsules formulated from an emulsion of fish oil and an enteric coating suspended in a basic solution, preferably a 25% solution of ethylcellulose in ammonium hydroxide. This emulsion, suspended in the basic solution, is atomized into an acidic solution using an inert gas such as nitrogen or argon. As with Lim, only a single coating is provided over the fish oil.

As regards the proposed combination, Kantor is as unrelated to Francois as is Lim, for the same reasons as pointed out above with respect to Lim. As regards the proposed combination of Lim in view of Kantor, even though these are of the same or related arts, the respective operations of Lim and Kantor are so different from one another that applicants do not see how they could possibly be reasonably combined with one another, without first reading applicants' specification.

In this latter regard, the rejection says that Kantor teaches rinsing the beadlets in an acid solution and drying, but applicants do not see that this is so. Instead, Kantor **produces** the microcapsules by injecting a basic solution into an acid solution. Kantor does not suggest washing already formed microcapsules in an acidic solution, and thus Kantor does not even teach that for which the PTO

relies upon it. Therefore, even if the combination as proposed were obvious, it would not reach the claimed subject matter.

The fourth reference relied upon in combination with the other three is Patel which, like Francois, deals with encapsulating solids rather than liquids. Patel discloses a great variety of possibilities, but primarily appears to disclose the provision of a complex coating over a solid carrier, the complex coating including the pharmaceutical active ingredient and one or more of surfactants and/or lipophilic components. Applicants do not see what relationship Patel has to either the present invention or to Lim.

Accordingly, applicants do not see what the person skilled in Lim's art, such person seeking to improve Lim in any way, would learn from Patel, even if such skilled person were charged with knowledge of Patel. Would such person learn that the active component should be put in the coating over a solid carrier, which appears to be a primary teaching of Patel? If so, Patel would be a teaching **away from** the present invention, and any modification of Lim by a teaching of Patel would result in something very different from what is claimed.

Applicants respectfully submit that the proposed combination would not have been obvious for the reasons given

above. Moreover, even if a person of ordinary skill in the art were to attempt to combine all four references together, the claimed subject matter would not have been obtained. No reference insofar as is known provides three coatings. There would have been no motive or incentive for doing so. Indeed, the only thing which might be predicted from providing three coatings as claimed would be to increase the cost and produce microcapsules which would be believed to have decreased bioavailability because of a plurality of coatings. To the contrary, applicant's microcapsules have a relatively high lipophilic compound content with improved bioavailability and stability. There is no reasonable expectation of such results from a consideration of the references.

To briefly summarize, please note that although generally the concept of encapsulating oils and oil soluble substances in microcapsules is known e.g. from the acknowledged prior art, the present invention is aimed at solving technical issues which had not yet been resolved in all of the prior art documents. Thus, it is unreasonable for a person skilled in the art to have followed the diverse procedures outlined in Lim, Francois, Kantor and Patel and to then have come up with a novel procedure, characterized by three coating layers and an extremely small particle size.

Furthermore, since none of the prior art documents teaches that a high lipophilic content can be achieved, a person skilled in the art would have no motivation to combine the teachings of the four prior art documents, as such person would be in no position to foresee the improved features of the product, namely superior bioavailability and stability, and significantly increased active ingredient content.

Regarding claims 2 and 3, the rejection states, and the PTO thus agrees, that the particle size described by Francois "is not near" that of the current invention. The significantly smaller particle size inherently affects the bioavailability of the lipophilic compound and results in a superior composition to that of the prior art.

Claim 13 is patentable for the same reasons as claim 1, the applicants hereby respectfully repeating by reference the comments made above.

As regards the various dependent claims, they are patentable **at least** for the reasons that they depend from and thus incorporate the subject matter of the independent claims from which they depend. In at least some cases, the dependent portions of these claims add additional non-obvious features. At the present time, however, applicants need not address these additional features, but respectfully reserve the right

to do so in the future if such should become necessary or desirable.

Applicants respectfully request withdrawal of the rejection based on §103.

New claim 25 has been added above. This new process claim parallels claim 1 in general, but adds some additional details including features from claims 2, 8 and 12. Claim 25 is patentable for the same reasons as claim 1, as pointed out above.

The prior art documents of record and not relied upon have been noted along with the implication that such documents are deemed by the PTO to be insufficiently pertinent to warrant their applications against any of applicant's claims.

Favorable reconsideration and allowance are earnestly solicited.

Respectfully submitted,

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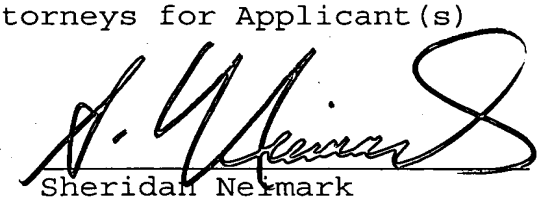
Attorneys for Applicant(s)

By 
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Injectable Microencapsulated Islet Cells as a Bioartificial Pancreas

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ABSTRACT

Rat islets encapsulated in semipermeable membranes remained viable in culture for 4 months. Multiple allotransplants of islets encapsulated in alginate–polylysine–polyethyleneimine membranes restored normoglycemia in recipient diabetic rats for most of a 90-day experimental period. Each individual transplant restored normal fasting plasma glucose levels for 15–20 d. The failure of the encapsulated islets was caused by an inflammatory response induced by polyethyleneimine. In contrast a single transplant of islets encapsulated in a biocompatible alginate–polylysine–alginate membrane restored normoglycemia in recipient animals for up to 10 months. Capsules with intact membranes and containing viable islets were recovered from the abdominal cavity 5 months post-transplantation. SEM studies on capsule membranes revealed essentially smooth surfaces. Differences between wet and dry wall thicknesses indicated that the membrane is a hydrogel, 4.00 ± 0.28 μm thick in an aqueous environment.

The clinical potential of transplanting cells encapsulated in biocompatible semipermeable hydrogel membranes is demonstrated by this study.

Index Entries: Microencapsulation, of islet cells; islet transplantation, microencapsulation in; bioartificial pancreas; biocompatible microencapsulated islets; alginate, in encapsulated islet support; poly-L-lysine, in encapsulated islet support; polyethyleneimine, in encapsulated islet support; islets, microencapsulated; pancreas, microencapsulated islets as an artificial.

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INTRODUCTION

The major obstacle to islet transplantation as a treatment for diabetes is rejection of the transplanted tissue by immune rejection. One approach to overcome this problem is the introduction of a semipermeable physical barrier between the transplanted islets and the host's immune system. A limited amount of success has been achieved in experimental animals using hollow fiber devices (*1–3*). Microencapsulation of islets within semipermeable membranes is an attractive alternative. Microbial cells, yeast, chloroplasts, plant chloroplasts, mitochondria, and enzymes have been immobilized or entrapped using calcium alginate gels (*4–6*).

Lim and Sun (*7*) successfully microencapsulated islets using alginate gel, poly-L-lysine, and polyethyleneimine. They demonstrated also that the encapsulated islets could restore normoglycemia in diabetic rats when injected into the peritoneal cavity.

This paper describes long-term in vitro and in vivo studies with islets encapsulated using two different membrane formulations and demonstrates that transplanted islets encapsulated in a biocompatible membrane can survive for up to 10 months.

MATERIALS AND METHODS

Isolation of Islets

Islets were isolated from out-bred Wistar rats (body weight, 200–250 g) using collagenase digestion techniques (*8,9*). They were hand-picked and cultured for 1–3 d at 37°C in tissue culture medium CMRL-1969® supplemented with 7.5% bovine fetal serum and 300 mg/dL glucose (*10*).

Microencapsulation of Islets in Alginate–Polylysine–Polyethyleneimine Membranes

The method used was reported previously by Lim and Sun (*7*). Islets were suspended in 0.8% sodium alginate (Sigma, St. Louis) and 0.85% NaCl at a concentration of 1×10^3 islets/mL. Islet-containing droplets formed by syringe pump extrusion gelled upon collection in 1.5% $CaCl_2$. These gels were washed with 25 mL each of 0.1% CHES [2-(*N*-cyclohexylamino)ethane-sulfonic acid] and 1.0% $CaCl_2$ and then suspended in 25 mL of a 0.02% poly-L-lysine (Sigma, mw 25,000–70,000, PLL) solution for 6 min. After washing with 1.0% $CaCl_2$, the capsules were coated with polyethyleneimine (PEI) by suspension in a 0.2% solution of PEI (Polysciences, mw 40,000–60,000) for 4 min. Following washing steps with $CaCl_2$ (1%) and NaCl (0.9%), the capsules were suspended in isotonic sodium citrate for 6 min to liquify the unbound alginate within the capsules.

Microencapsulation of Islets in Alginate–Polylysine–Alginate Membranes

To produce more biocompatible and durable microcapsules, some critical changes were made in the microencapsulation procedure originally reported by Lim and Sun (7). The outer polyethyleneimine membrane was omitted and replaced by a more biocompatible outer alginate membrane. The strength of the polylysine membrane was enhanced by increasing its thickness.

Islet Culture

Both microencapsulated and unencapsulated islets were cultured at 37°C in Medium CMRL-1969 supplemented with 7.5% fetal bovine serum and 300 mg/dL glucose. During long-term cultures the medium was replaced weekly and the glucose concentration alternated between 50 and 300 mg/dL.

Electron Microscopy Studies

For electron microscopy, encapsulated rat islets were fixed in 2.5% glutaraldehyde, post-fixed in 1% OsO_4, dehydrated and embedded in an Epon-Araldite mixture. Ultrathin sections were cut and stained with uranyl acetate and lead citrate.

Histochemical Studies

Encapsulated islets and tissue segments were fixed in Bouin's solution and processed for light microscopy. Sections were stained with aldehyde thionin, hematoxylin, and eosin.

Allotransplantation Experiments

Diabetes was induced in out-bred Wistar rats by iv injection of streptozotocin (65 mg/kg body weight). Blood samples for fasting plasma glucose concentration were collected by bleeding from the orbital sinus. A Beckman Glucose Analyzer was used for glucose measurement. Diabetes was confirmed by the development of persisting fasting plasma glucose levels in excess of 350 mg/dL. Microencapsulated islets, suspended in saline, were implanted in the peritoneal cavity of the diabetic rats using a cannula attached to a 10-cc syringe.

Surface Finish Evaluation of Capsules by Scanning Electron Microscopy (SEM)

Microcapsules were placed directly on an aluminum SEM stub and dried in a vacuum oven at 60°C for 1.5 h. The samples were coated with approximately 250 Å of gold and inserted into the SEM. Both the interior

and exterior surfaces of the microcapsules were examined. The SEM was also utilized to make microcapsule wall thickness measurements.

Wall Thickness and Uniformity of Microcapsules

Microcapsules were placed on aluminized, front-surface mirrors and dried at room temperature for 24 h. The dry-wall thickness was measured by interferometry. The wet-wall thickness and wall uniformity of the microcapsules was measured by the image-shearing method. Four wall-thickness measurements were made at the equator of each capsule.

RESULTS

Electron Microscopy

An electron microscopy study of encapsulated islets revealed that the structural integrity of the cells was maintained. The typical granulation of both α-cells (Fig. 1a) and β-cells (Fig. 1b) was clearly seen in encapsulated islets which were maintained in culture for 90 d.

Histology of Microencapsulated Islets

Islets encapsulated in the alginate–polylysine–polyethyleneimine membrane were cultured for 90 d before being processed for light microscopy. The islets remained relatively intact and showed uniform and even staining with aldehyde thionin throughout all islet cross-sections (Fig. 2a). Islets encapsulated in the alginate–polylysine–alginate membrane showed good β-granulation until at least 135 d (Fig. 2b).

Allotransplantation Experiments with Alg–PLL–PEI Capsules

Two rats with streptozotocin-induced diabetes received multiple intraperitoneal transplants of approximately 3000 encapsulated islets as described in Methods. The blood glucose levels, urine volumes, and body weights were monitored at regular intervals. The initial transplant lowered the blood glucose concentration from 375 mg/dL to control values (75–150 mg/dL) within 2 d. When the animals returned to the diabetic state, a second transplant of encapsulated islets was performed and the blood glucose levels were again lowered to within the control range. This pattern was repeated with three further transplants. On each occasion, blood glucose concentrations remained below diabetic levels for 15–20 d (Table 1). A control group of diabetic rats receiving no transplants or transplants of empty capsules had blood glucose levels higher than 350 mg/dL throughout this study. Fluctuations in blood glucose concentration of the animals receiving transplants were reflected by alterations in the daily urine output, the volume decreasing with a lowe

ing of the blood glucose concentration and increasing as the glucose level increased.

The body weight of animals receiving islet transplants increased from 230 and 260 g to 285 and 320 g, respectively. In contrast, the body weights of the control diabetic animals did not change significantly (± 10 g) over the same period of time.

As is usual in streptozotocin-induced diabetes, the control diabetic animals all developed eye cataracts within 12 weeks of the onset of diabetes. In contrast, the eyes of the two animals receiving multiple islet implants remained cataract-free.

The animals were sacrificed 20 d after the final transplants. No free capsules were found in the abdominal cavity. Sections of liver, pancreas, kidney, and the peritoneum were taken for histology studies. Foreign body granulosa were observed on all tissues. The capsular membranes were completely surrounded by giant cells and fibrous tissue (Fig. 3). No islets were found in any of the sections examined. Implanation of empty capsules in control animals resulted in a similar foreign body reaction within 10–20 d.

Allotransplantation Experiments with Alginate–Polylysine–Alginate Capsules

A series of five diabetic rats received intraperitoneal allografts of 4.5×10^3 islets encapsulated in alginate–polylysine–alginate membranes. Normoglycemia (fasting plasma glucose < 200 mg/dL) was restored within 2 d and persisted for up to 10 months (Fig. 4). Of the five animals receiving transplants, four remained normoglycemic for > 77 d and three of these for > 120 d. One animal is still normoglycemic 150 d post-transplant, a second animal still has fasting plasma glucose levels < 200 mg/dL 310 d post transplantation. Capsules were recovered from the peritoneal cavity of one recipient 156 d post-transplantation. The capsules had some host cells attached to the outside surface of the membrane but were still intact. When maintained in culture these encapsulated islets secreted low levels of insulin into the culture medium.

All recipient animals gained weight steadily during their normoglycemic period. The mean weight gain was 116 ± 16.5 g (mean ± SEM). None of the animals developed eye cataracts.

Surface Finish, Wall Thickness, and Uniformity of Alginate–Polylysine–Alginate Capsule Membranes

Surface finish evaluation by scanning electron microscopy (SEM) revealed essentially smooth interior and exterior capsular surfaces. Although some samples were observed to have "pebble-like" surface features about 0.1 μm in size and 0.15 μm apart (Fig. 5a), this could have been caused by the electron beam of the SEM. Utilizing the SEM, dry

Fig. 1. Electron micrographs of sections of an encapsulated islet that hac been maintained in culture at 37°C for 90 d: (a) section showing α-cell with glucagen granules (×9000). (b) section showing β-cell with insulin granules (×14,000).

capsule walls thicknesses ranging from 0.2 to 0.7 μm were observed (Fig 5b), although it was not always clear whether or not a single wall was being measured. Using interferometry, the two-wall thickness of dried microcapsules was determined to be 0.57 μm ± (mean ± 0.11 μm SD $N = 10$). Interferometric pictures demonstrated that there was very little variation in the two-wall thickness of individual dried microcapsules.

The "wet" wall thickness and wall variation of 10 randomly selected capsules, determined by the image shearing method, was found to be 4.00 ± 0.28 μm(± SD). The average capsule wall variation, obtained by averaging the 10 standard deviations obtained from the four thickness measurements of each microcapsule, was determined to be 0.32 μm. Only small differences were observed from sample to sample.

Fig. 1B.

DISCUSSION

The medical applications of immobilized enzymes have been established by Chang et al. (*11–13*), who also first proposed the possible application of microencapsulation for islet cells (*14*). Lim and Sun (*7*) demonstrated that rat islet cells, encapsulated in alginate–polylysine–polyethyleneimine membranes, could be maintained in culture for several months and when transplanted intraperitoneally into diabetic rats, restored normoglycemia for 15–20 d. The histology results in the Lim and Sun study and those described above showed that encapsulated islets could be maintained in a viable state in culture for at least 4 months and still retain a normal degree of β-granulation. The electron microscopy studies illustrated that the structural integrity of the α- and β-cells were retained during long-term culture. It has previously been reported by our laboratory that encapsulated islets continued to secrete insulin into the culture medium in response to a glucose challenge during a 2-month incubation period (*15*). Working with erythrocytes Pilwat et al. (*16*) also demonstrated that cells entrapped in a crosslinked alginate ma-

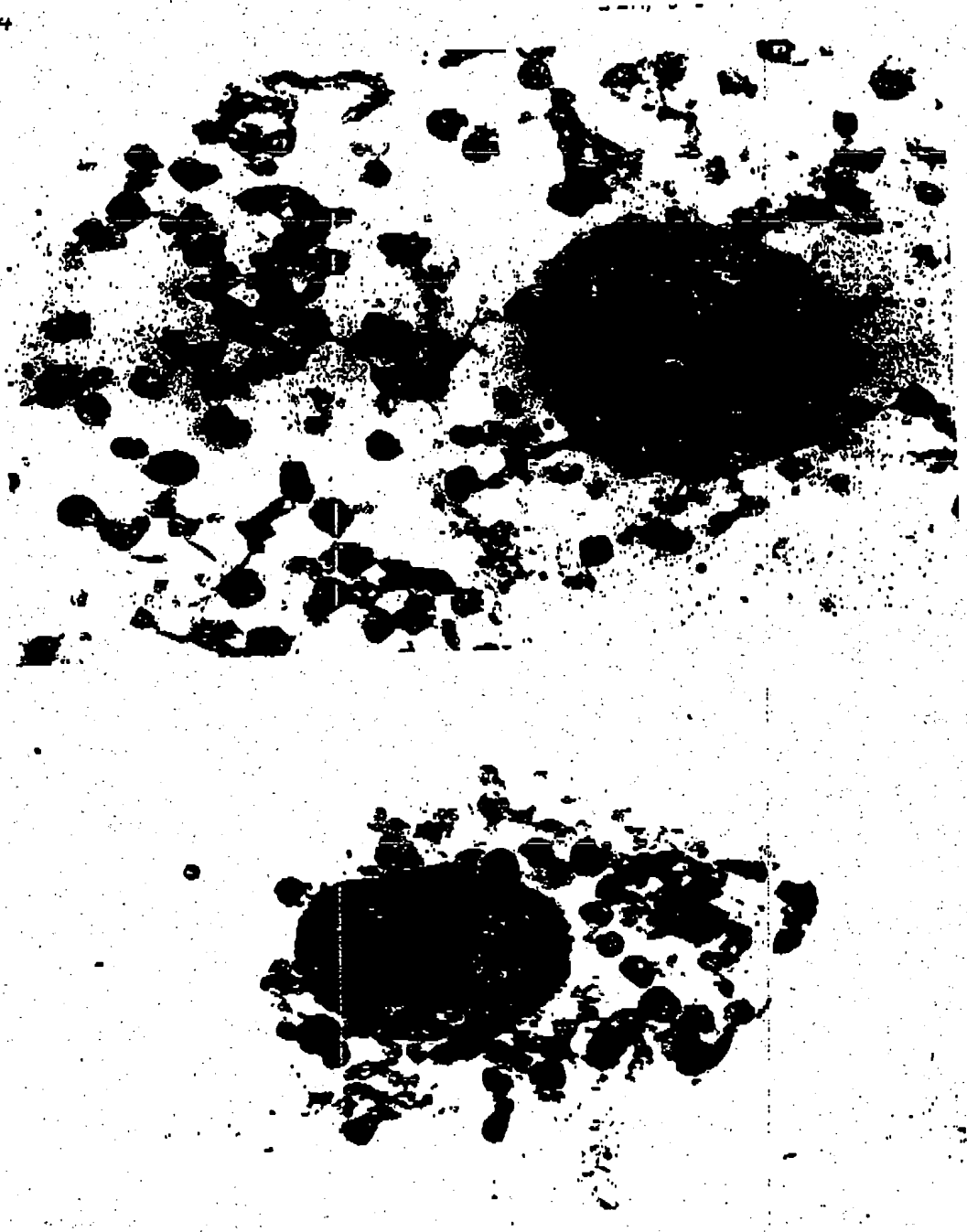

Fig. 2. Light micrographs of encapsulated pancreatic islets: Top: islets in alginate–polylysine–polyethyleneimine membranes after 90 d in culture; Bottom: islets in alginate–polylysine–alginate membranes after 135 d. Aldehyde thionin stain. Magnification 30×.

TABLE 1
Duration of Normoglycemia and Weight Increase in Diabetic Rats Following Multiple Transplants of Encapsulated Islets

	Duration of normoglycemia, d					
	Transplant No.					
Animal	1	2	3	4	5	Wt increase, g
A	16	16	18	17	18	55
B	15	16	17	20	13	60

Fig. 3. Light micrograph of foreign body granuloma in a diabetic rat that had received 5 ip transplants of 3×10^3 microencapsulated islets. The animal was sacrificed 90 d after the initial transplant and the liver processed for histochemical staining. A capsule membrane (arrow) can be seen completely surrounded by giant cells and fibrous tissue. Magnification 7.5×.

trix could be stored for substantially longer periods than those kept in suspension.

The allograft experiments with islets encapsulated in alginate–polysine–polyethyleneimine membranes demonstrated that in addition to a single intraperitoneal transplant of 3×10^3 islets restoring normoglycemia for 2–3 wk as reported by Lim and Sun (*7*), repeated transplants maintained the animals in a near-normoglycemic state during the 90-d experimental period. Each time the animals became

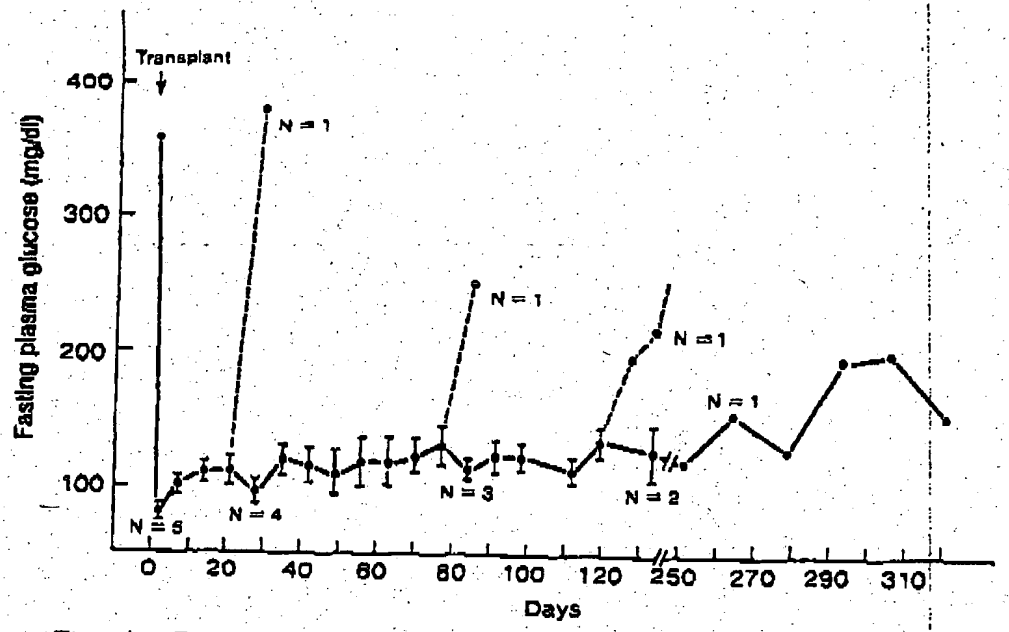

Fig. 4. Fasting blood glucose levels in rats transplanted with encapsulated islets. Male Wistar rats rendered diabetic by iv injection of streptozotocin (65 mg/kg) were transplanted with approximately 4.5×10^3 islets encapsulated in alginate–polylysine–alginate membranes. Samples for glucose assay were collected by bleeding through the orbital sinus. The points are mean values and the bars represent the SEM.

hyperglycemic an additional transplant restored normal fasting glucose concentrations for a period lasting at least as long as the previous transplant. This suggests that a simple immunologic rejection of the transplanted tissue was not occurring. Histology studies revealed the presence of foreign body granulosa in the tissues of the host animals. Capsule membranes were engulfed by giant cells and fibrous tissue, indicating that the capsules were not biocompatible. The composition of the capsule membrane was subsequently modified to improve its biocompatibility. The thickness of the polylysine layer was increased and the outer polyethyleneimine layer replaced by inert alginate. A single allotransplant of islets encapsulated in this modified membrane restored normoglycemia in diabetic recipients for up to 10 months.

The modifications to the composition of the capsule membrane increased the biocompatibility of the capsules very significantly. With the alginate–polylysine–alginate membrane, capsules were recovered from the peritoneal cavity 5 months post-transplantation. In comparison, with the original alginate–polylysine–polyethyleneimine capsules, no free capsules were found; a rapid foreign body reaction was induced within 20 d. The SEM studies showed that the alginate–polylysine–alginate cap

sules have a relatively smooth surface. The wet wall thickness is appr ximately 14 times greater than the dry-wall thickness indicating that the capsule wall is a hydrogel containing approximately 90% water. Hydrogels are receiving considerable attention as candidates for good biocompatible materials (*17–19*). The low interfacial tension between the swollen gel surface and the aqueous biological environment minimizes protein interaction. This interaction (*19*) may serve as a trigger mechanism for rejection mechanisms. The soft rubbery consistency of most hydrogels may also contribute to their biocompatibility by reducing frictional irritation to surrounding tissues (*20*).

CONCLUSION

Islets encapsulated in a biocompatible and durable hydrogel membrane composed of polylysine sandwiched between two alginate layers can be transplanted into the animal body and survive for at least several months. Such capsules have a great clinical potential for the treatment of diabetes and other hormone or enzyme replacement therapies.

ACKNOWLEDGMENTS

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